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Short communication

# A specific cathepsin-L-like protease purified from an insect midgut shows antibacterial activity against gut symbiotic bacteria

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ABSTRACT

Because gut symbiotic bacteria affect host biology, host insects are expected to evolve some mechanisms for regulating symbiont population. The bean bug, *Riptortus pedestris*, harbors the *Burkholderia* genus as a gut symbiont in the midgut organ, designated as the M4 region. Recently, we demonstrated that the lysate of M4B, the region adjacent to M4, harbors potent antibacterial activity against symbiotic *Burkholderia* but not to cultured *Burkholderia*. However, the *bona fide* substance responsible for observed antibacterial activity was not identified in the previous study. Here, we report that cathepsin-L-like protease purified from the lysate of M4B showed strong antibacterial activity against symbiotic *Burkholderia* but not the cultured *Burkholderia*. To further confirm this activity, recombinant cathepsin-L-like protease expressed in *Escherichia coli* also showed antibacterial activity against symbiotic *Burkholderia*. These results suggest that cathepsin-L-like protease purified from the M4B region plays a critical role in controlling the population of the *Burkholderia* gut symbiont.



## 1. Introduction

Many insects are known to possess symbiotic bacteria within their cells, tissues, and guts (Douglas, 2014; Ferrari and Vavre, 2011). These insect-symbiont associations have been established for a long time and are known to affect the biology of host insects in various ways. One factor expected to affect the host's biology is the size of the symbiont population. The level of the symbiont population affects the host's fitness, the fidelity of vertical transmission, and the intensity of reproductive aberrations (Koga et al., 2003; McGraw et al., 2002; Mouton et al., 2004). Therefore, it is assumed that host insects may have systems to control and maintain their symbiont populations within an optimal range. Several studies supportive of insect control over symbiont populations have been reported: control of *Buchnera* symbionts by lysozyme in aphids (Nakabachi et al., 2005; Nishikori et al., 2009), reactive oxygen species production for controlling *Wolbachia* in mosquitoes (Brennan et al., 2008), and antimicrobial peptides (AMPs) for controlling primary endosymbionts in *Sitophilus* weevils (Login et al., 2011). However, these studies have mostly focused on the intracellular symbionts of insects, whereas the mechanisms for controlling the extracellular gut symbionts of insects are poorly understood.

The bean bug, *Riptortus pedestris* (Hemiptera: Heteroptera: Alydidae), possesses a specialized symbiotic organ in a posterior midgut region (named M4 region), where numerous crypts harbor extracellular β-proteobacterial symbionts of the genus *Burkholderia* (Kikuchi et al., 2005). This symbiont, which is orally acquired by second instar *Riptortus* nymphs from the environment every generation, is easily cultivable and genetically manipulable (Kikuchi et al., 2007, 2011; Kim et al., 2013a). Hence, the *Riptortus-Burkholderia* symbiotic system has been recognized as a promising experimental model to study insect-microbe symbioses at the molecular level. Using this model system, we recently reported unexpected observation that a specified midgut region (M4B, adjacent region of M4) in host *Riptortus* may play a role in controlling the *Burkholderia* gut symbiont (Kim et al., 2013a). As evidence, we demonstrated that the M4B midgut region showed specific and potent antibacterial activity against the symbiotic *Burkholderia* and that the antibacterial activity was induced by the presence of gut symbiont, suggesting that this unrecognized insect midgut organ may be involved in controlling the gut symbiont

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population (Kim et al., 2013a). However, molecular studies regarding the identification of the molecule(s) showing antibacterial activity against the symbiotic *Burkholderia cells* were not addressed in our previous study.

Here, 25 kDa protein purified from the M4B lysate of symbiont-harboring fifth instar *Riptortus* nymph showed antibacterial activity against *Burkholderia* gut symbiont. This protein was determined as cathepsin-L-like protease of host midgut. The recombinant protein expressed in *Escherichia coli* also showed the same antibacterial activity as naïve purified cathepsin-L-like protease, providing evidence that insect gut cathepsin-L-like cysteine protease regulates the population of gut symbiotic bacteria.

## 2. Materials and methods

### 2.1. Insect rearing

*R. pedestris* bean bugs were reared in our insect laboratory at 28 °C under a long-day regime of 16 h light and 8 h dark, as described previously (Kim et al., 2014). Briefly, nymphal insects were reared in clean plastic containers supplied with soybean seeds and DWA (distilled water containing 0.05% ascorbic acid). Upon reaching adulthood, the insects were transferred to larger containers in which soybean plant pots were placed for feeding and cotton pads were attached to the walls for egg laying. Eggs were collected daily and transferred to new cages for hatching.

### 2.2. Burkholderia symbiont inoculation

The *Burkholderia* symbiont strain RPE75, which is a spontaneous rifampin (RIF)-resistant mutant derived from the strain RPE64 (Kikuchi et al., 2011), was cultured at 30 °C in YG-RIF medium (YG medium [0.5% yeast extract, 0.4% glucose, and 0.1% NaCl] containing 30 μg/ml rifampin). The inoculum solution was prepared by suspending mid-log-phase cultured *Burkholderia* cells in double distilled water (DWA) at a concentration of $10^7$ cells/ml. Newly molted second-instar nymphs were provided with wet cotton balls soaked with the inoculum solution. After the insects had been fed with the inoculum solution for 2 days, fresh, sterile DWA was provided to the insects instead of the inoculum solution (Kim et al., 2013b). A transgenic *Burkholderia* strain labeled with green fluorescent protein (GFP) was constructed and fed in the same way as wild-type *Burkholderia* cells, as described previously (Kikuchi and Fukatsu, 2014).

### 2.3. Purification of cathepsin-L-like protease from M4 midgut lysate via gel filtration column

M4 midgut regions were collected from 150 fifth instar nymphs in buffer A (20 mM Tris, 100 mM NaCl, pH 7.0) and homogenized with a plastic bar. The supernatant was collected by centrifugation and filtered with a 0.45 μm filter as a M4B lysate. The lysate then was loaded on a TSKG3000SWXL HPLC column (7.8 mm × 30 cm, TOSOH, Japan) equilibrated with buffer A and eluted with the same buffer A at a 0.5 ml/min flow rate. Each fraction was analyzed on 13% SDS-PAGE under reducing conditions.

### 2.4. Plasmid construction, protein expression and purification

The gene encoding procathepsin-L-like protease was amplified by PCR using cDNA from the M4B of *R. pedestris* fifth instar nymphs. The amplified products were ligated into the pRSET-A (Life Technologies) vector, and the resulting vector was introduced into the *E. coli* BL21 (DE3) strain (NEB Inc.). The induced bacterial cell cultures were grown to an A600 of 0.6 and induced for the production of Histagged cathepsin-L-like protease by the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) overnight at 30 °C with shaking at 180 rpm. Because recombinant protein was expressed in inclusion body, the cathepsin-L-like protease was recovered from the insoluble fraction after cell lysis. The insoluble pellet from a 400 ml of culture was solubilized with solubilization buffer B (30 ml of 100 mM Tris-Cl, pH 8.5, 1 mM dithiothreitol, containing 8 M urea). The recombinant protein bound to a Ni-Sepharose column (GE healthcare Inc.) was washed with buffer C (50 ml of 100 mM Tris, pH 7.0, 300 mM NaCl, 15 mM imidazole, 1 mM dithiothreitol, 1 X Protease inhibitor cocktail (Sigma Aldrich), and 1% Triton X-100) after the column was eluted with buffer C containing 500 mM imidazole. The purity of the soluble cathepsin-L-like protease form was examined by 15% SDS-PAGE, and the N-terminal sequence of the purified recombinant protein was determined by the gas-phase Edman method.

### 2.5. Measurement of the protease activity of recombinant cathepsin-L-like protease

To determine the amidase activity of the expressed protease, two different synthetic substrates, Z (carbobenzyloxy)-Phe-Arg-MCA (methylcoumaryl-7-amide) and Z-Arg-Arg-MCA, were used as described previously (Zhang et al., 2003). These substrates were dissolved in dimethyl sulfoxide or dimethylformamide according to the manufacturer's instructions. Enzyme reactions were performed in 0.5 ml of buffer D (20 mM citrate buffer, pH 6.0, containing 50 μM substrate, 75 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol) with the addition of the expressed protease. After incubation of the mixture at 27 °C for 10 min, 700 μl of 17% (v/v) acetic acid was added to terminate the enzyme reaction. The specific amidase activity of the eluate solution was detected by a fluorescence spectrophotometer at λex = 380 nm and λem = 460 nm. As a control, 10 μl of buffer D was prepared to check the amidase activity as described above.

### 2.6. CFU assay for measuring antimicrobial activity

The symbiotic *Burkholderia* strain RPE75 and GFP-labeled *Burkholderia* strain RPE75 were freshly isolated from the M4 region as previously described (Kim et al., 2013b; Kikuchi and Fukatsu, 2014). These bacterial cells were washed and diluted with phosphate buffer (PB) to 500 to 1000 CFU per 100 μl M4B whole lysate was prepared by homogenizing M4B samples dissected from ten fifth-instar nymphs in 100 μl PB, and the lysate was serially diluted with PB. Each sample, consisting of 10 μl of the lysate and 10 μl of the bacterial suspension, was incubated at room temperature for 15 min, and the samples were spread onto YG-RIF agar plates, cultured for 2 days, and subjected to colony counting.

## 3. Results and discussion

### 3.1. Purified cathepsin-L-like protease from of the gut M4B lysate showed antibacterial activity against symbiotic Burkholderia cells

In our previous study, the antimicrobial activity of the host gut M4B region was detected in symbiotic host insects but not in aposymbiotic host insects, and this activity reached its highest level in the fifth instar nymph insects (Kim et al., 2013a). To address which molecule from the M4B region is responsible for the observed antibacterial activity against gut symbiotic bacteria, we collected the midgut M4B regions of fifth instar nymphs harboring gut *Burkholderia* symbiont and prepared M4B lysate; then, M4B lysate was loaded on a TSK SW3000 gel filtration column. Approximately nine peaks were distinguished, as shown in Fig 1A.